XLIII. BARGER'S MICROSCOPICAL METHOD OF DETERMINING MOLECULAR WEIGHTS.

PART II. ITS APPLICATION TO CASEINOGEN.

By KUMAO YAMAKAMI.

From the Lister Institute of Preventive Medicine, London.

(*Received April 12th, 1920.*)

A. The molecular and ionic concentration of alkali caseinogenate solutions of neutral and alkaline reaction.

This work has been undertaken to study the osmotic concentration of alkali caseinogenate solutions. T. B. Robertson [1909, 1918, p. 336] and T. B. Robertson and T. C. Burnett [1909] have investigated the cryoscopic behaviour of dissolved caseinogenates of alkalies and alkaline earths (NH_4OH, KOH, LiOH, $Ca(OH)_2$ etc.) and found that:

"Keeping the concentration of casein constant and increasing the concentration of base bound by the caseinogen results in a proportionate increase in the observed depression, in other words, the molecular and ionic concentration of caseinate solutions is conditioned by the combined alkali. On the other hand, the increasing of the quantity of dissolved casein does not alter the observed depression in any appreciable degree when casein is dissolved in the solution of base in the proportion of 80.10^{-5} gram equivalent or 50.10^{-5} gram equivalent base per gram casein."

From these experimental results they concluded that each equivalent of combined base yields the same number of protein ions derived from the splitting of successive .CO.NH groups, and suggested that "if this relation were maintained for other concentrations, then at zero concentration of combined base, if casein were soluble under such conditions, the freezing point depression due to dissolved casein would be zero. The possibility is therefore indicated that base- and acid-free protein may exert an immeasurably small osmotic pressure, probably owing to the polymerisation of protein as the uncombined protein is set free."

When I was in the laboratory of Toronto University, it was suggested that I should apply Barger's microscopical method of determining molecular weights to caseinogen. Accordingly I investigated the osmotic concentrations of alkali caseinogenate solutions employing caseinogen which was prepared by a colleague, but I could not demonstrate the parallelism between the

molecular ionic concentrations of caseinogenate solution and the concentration of the base in which the caseinogen was dissolved.

Prof. Robertson suggested that this might be due to the impurity of the caseinogen employed, and this proved to be the case. For, when I repeated the experiments at the Lister Institute, London, with "Hammarsten casein" (Kahlbaum) consistent results were obtained.

The molecular and ionic concentrations of Na-caseinogenate solutions have been observed to be absolutely dependent upon the concentration of NaOH, not only when the proportion of base to caseinogen is between $80 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents base per g. caseinogen, but also for all other ratios between $180 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents.

Experiment 1. "Hammarsten casein" (Kahlbaum) was purified according to the directions of Robertson [1918, p. 39] and was dissolved in NaOH solutions of varying concentrations, changing the amount of caseinogen, so that the ratio of caseinogen to base varied between $180 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents base per g. caseinogen and the molecular and ionic concentrations of these caseinogenate solutions were determined by means of Barger's method, employing urea as the standard substance.

Unfortunately there is at present no good test for the purity of caseinogen, but its degree of purity in my experiments is indicated by the following results of the tests which are generally applied.

1. A perfectly white powder which is absolutely dry.
2. Its solution is colourless.
3. The percentage of ash is 0·11.
4. The solution in alkali in the proportion of $80 . 10^{-5}$ g. equivalent is just neutral to phenolphthalein.
5. The solubility in 5 % NaCl solution is less than 2 %.

If van Slyke and Bosworth's statement [1913] that the usual caseinogen preparations obtained from chemical supply houses are generally contaminated with water-insoluble calcium caseinogenate (monobasic caseinogenate) and calcium phosphate is true, the directions for purification given by Robertson cannot be very effective, but the solubility in NaCl solution as well as the content of ash in the caseinogen preparation employed in my experiments indicate that it is sufficiently pure for the purpose.

The purity of the urea employed as standard had been checked by determining the molecular weights of other pure substances such as cane-sugar, lactose, etc. with its aid.

The first measurement of the drops was made 30–40 minutes after the preparation of the tubes and the second determination after standing for 24 hours in a cold room.

The caseinogenate solutions in these experiments had generally small molecular-ionic concentrations, consequently the change of the drops was very slight even when the tubes were exposed to ordinary room temperature. Nevertheless the tubes were kept cool in the vestibule of a cold room where

the temperature was about 10°–11° C., because it was feared that the decomposition of caseinogen by micro-organisms which cannot be prevented from entering the solution, might take place more easily at room temperature.

The technique employed is exactly the same as described in Part I [Yamakami, 1920], the directions of Barger being strictly followed. In the first determination the absolute measurements of the drops and the changes in them are given by way of illustration. In all the remaining determinations only the concentrations of the solutions in the limiting tubes are given.

In the first column of the table, the molar concentration of the urea solutions is given, and the drops denoted by odd numbers are those of the caseinogenate solutions.

Table I.

(*a*) NaOH = *N*/20, caseinogen = 10 %; alkali: caseinogen = 50 . 10^{-5} g. eq.: 1 g.

Urea	I	II	III	IV	V
M/12	221 – 7	384 + 7	235 – 7	359 + 11	303 – 10
M/14	251 – 6	279 + 6	270 – 15	331 + 7	209 – 9
M/16	402 – 1	676 + 2	175 – 4	754 + 4	608 – 4
M/20	388 + 2	555 – 1	490 + 1	612 ± 0	224 ± 0
M/25	504 + 12	841 – 1	785 + 5	670 + 4	492 + 2

Limits = 1/16 – 1/20 *M*.

(*b*) NaOH = *N*/20, caseinogen = 8 %; alkali: caseinogen = 62 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*c*) NaOH = *N*/20, caseinogen = 6·2 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*d*) NaOH = *N*/20, caseinogen = 4 %; alkali: caseinogen = 125 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*e*) NaOH = *N*/20, caseinogen = 2·78 %; alkali: caseinogen = 180 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*f*) NaOH = *N*/20·8, caseinogen = 6 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/17·5–1/20 *M*.

(*g*) Caseinogen solutions containing respectively 50 . 10^{-5} and 80 . 10^{-5} g. equivalent alkali per g. caseinogen were compared directly.

I	II	III	IV	V
–2	–2	–3	–3	–2
–2	+1	+1	+1	+1
–3	–2	–2	–2	–1

The drops denoted by even numbers are those of caseinogen solution containing 50 . 10^{-5} g. equivalent alkali.

Table II.

(*a*) NaOH = *N*/25, caseinogen = 8 %; alkali: caseinogen = 50 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*b*) NaOH = *N*/25, caseinogen = 6 %; alkali: caseinogen = 66 . 7·10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*c*) NaOH = *N*/25, caseinogen = 5 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*d*) NaOH = *N*/25, caseinogen = 3·75 %; alkali: caseinogen = 106·6 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*e*) NaOH = *N*/25, caseinogen = 2·22 %; alkali: caseinogen = 180 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

Table III.

(*a*) NaOH = $N/33$, caseinogen = 6 %; alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Limit = 1/35 *M*.

(*b*) NaOH = $N/33$, caseinogen = 3·75 %; alkali: caseinogen = $57{\cdot}6 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/35–1/40 *M*.

(*c*) NaOH = $N/33$, caseinogen = 1·7 %; alkali: caseinogen = $182 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/30–1/35 *M*.

(*d*) NaOH = $N/50$, caseinogen = 4 %; alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

(*e*) NaOH = $N/50$, caseinogen = 3·7 %; alkali: caseinogen = $53 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

(*f*) NaOH = $N/50$, caseinogen = 2·5 %; alkali: caseinogen = $80 . 10^{-5}$ g. eq.: 1 g.
Limit = 1/40 *M*.

(*g*) NaOH = $N/50$, caseinogen = 2·2 %; alkali: caseinogen = $90 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

The results obtained in the above tables are so regular that detailed explanation and discussion are hardly necessary. The molecular and ionic concentrations of the caseinogenate solutions were always very nearly identical with the concentrations of the alkali solutions in which the caseinogen was dissolved, as long as the proportion of alkali to caseinogen was between $180 . 10^{-5}$ g. equivalent and $50 . 10^{-5}$ g. equivalent base per g. caseinogen.

The only thing I feared was that urea might combine chemically with the alkali of the caseinogenate solutions. But this, very probably, would not occur because the urea solution is very slightly acid to phenolphthalein, while the acidity of caseinogen is pretty strong. Thus the urea solution would not be able to attract the alkali which was combined with such a strong acid as caseinogen. This would be particularly true when the reaction of the caseinogenate solution itself was strongly acid to phenolphthalein. Moreover the regularity of the change of the drops suggests that no chemical reaction was taking place between the two solutions.

As to the effect of the impurities in the caseinogen upon the results obtained, the calcium caseinogenate which is alleged to contaminate most preparations of caseinogen would not exert much influence upon the molecular and ionic concentrations.

The possible impurities which must be taken into consideration in these experiments are crystalloid substances which have small molecular weights, because they would change the osmotic concentrations of the caseinogenate solutions to a considerable extent even if present only in small quantities. In order to get rid of this objection, I dialysed my caseinogenate solutions and convinced myself that the caseinogen used was free from crystalloid substances since no lowering of the molecular and ionic concentration of the caseinogenate solution resulted from dialysis. Reference will be made to these experiments later.

On one occasion, the direct comparison of caseinogenate solutions which contained varying amounts of caseinogen and alkali gave the same result,

and this indeed might well be expected, as in the last experiment recorded in Table I, in cases where the reaction of the two caseinogenate solutions compared did not differ very much. But when there existed large differences in the reactions of the two solutions, the change of the drops was quite irregular, so that no conclusion could be drawn from the experiment. This is probably due to the chemical attraction between the two solutions, which interferes with the regularity of movement of the solution from one drop to another because of the physical attraction of solute for solvent.

It is my belief that the observation made by Robertson and Burnett by means of the cryoscopic method is satisfactorily confirmed by my experiments in which Barger's method was employed.

Now, if this relationship between alkali, caseinogen and the molecular ionic concentration of the solution is maintained unchanged up to the highest percentage of caseinogen which is soluble in a given amount of alkali, then the largest molecular ionic weight, we can expect to obtain by Barger's method, must be dependent upon the solubility of caseinogen in the alkaline solution.

If caseinogen is soluble in the alkaline solution in the proportion of $25 . 10^{-5}$ g. equivalent of alkali per g. caseinogen then we should obtain 4000 as the molecular or ionic weight of caseinogen, if, on the other hand, the solubility of caseinogen is $12{\cdot}5 . 10^{-5}$ g. equivalent then the figure would become 8000 provided that the osmotic concentration of caseinogenate solution is always conditioned by the concentration of alkaline solution.

The solubility was, therefore, studied before I undertook the investigation of the molecular and ionic concentrations of the alkali caseinogenate solutions of acid reaction in which the proportion of alkali to caseinogen was smaller than $50 . 10^{-5}$ g. equivalent per g. caseinogen.

B. The solubility of caseinogen in alkali solution and the molecular weight of caseinogen calculated from the solubility or the combining capacity.

The solubility of caseinogen in alkali solution or "the combining capacity of casein at saturation of the base with casein, that is when the base has dissolved the maximum amount of casein which it will dissolve," as expressed by Robertson, has recently been stated to be $11{\cdot}4 . 10^{-5}$ g. equivalent alkali per g. caseinogen. This is based upon two experimental results, the one, obtained by Robertson, and the other, by van Slyke and Bosworth [1913], and it is supported by deduction from the formula for the lowering of the conductivity of alkali solution which is caused by dissolved caseinogen.

Robertson found that the relation between b_1, the concentration of alkali solution in which caseinogen is dissolved, and Δ, the lowering of its conductivity which is caused by the caseinogen, is expressed by the formula

$$\Delta \times 10^5 = 26{\cdot}880 b_1 - \frac{475{\cdot}800}{C} b_1^2 - 2898C.$$

Putting $\Delta = 0$, the concentration of alkali solution b_1 becomes $0{\cdot}000114C$. This proportion of alkali to caseinogen occurs at the point where the change in the conductivity of an alkaline solution, which is brought about by dissolving a given percentage of caseinogen, is zero, and coincides exactly with the value for the solubility of caseinogen which Robertson [1918] and van Slyke and Bosworth [1913] claim to have obtained experimentally.

Van Slyke and Bosworth, however, proved the fact that the caseinogenate of an alkaline earth is not soluble when the proportion of base to caseinogen is $11{\cdot}4 \cdot 10^{-5}$ g. equivalent: 1 g.; it is soluble in water only when the proportion is larger than $22{\cdot}5 \cdot 10^{-5}$. This fact seems to suggest very strongly that we should not identify the solubility of caseinogen in alkali solution with its combining capacity.

In other words, caseinogen may combine with alkali in the proportion of $11{\cdot}4 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen, as deduced from the above-mentioned formula, yet it does not follow that the product of combination must necessarily dissolve in water.

This suspicion with regard to the solubility of van Slyke and Bosworth's monobasic alkali caseinogenate is deepened when we carefully examine the experiments of the authors who claim $11{\cdot}4 \cdot 10^{-5}$ g. equivalent as the solubility as well as the combining capacity of caseinogen to alkali.

When they determined the solubility of caseinogen in alkali, they did not dissolve directly as much caseinogen as possible in alkali solution, but in both investigations a measured amount of caseinogen was dissolved in a known quantity of alkali capable of very easily dissolving the caseinogen taken and then the point was determined at which the first permanent precipitation of caseinogen appeared on neutralising the caseinogen solution with HCl, using the refractometric method (Robertson) or centrifuging (van Slyke and Bosworth). The value, which they call the solubility, is therefore the quantity of alkali used to dissolve the caseinogen minus the amount of alkali neutralised by HCl.

But it must be taken into consideration that the resulting solution of caseinogenate in the experiments of these authors is not a pure aqueous solution of alkali caseinogenate, but a caseinogenate solution containing alkali chloride. Thus the resulting solution in van Slyke and Bosworth's experiment contained almost 0·5 % alkali chloride.

The solubility of caseinogen in NaCl solution seems to be undeniable [Robertson, 1918, p. 88], although it is questioned by van Slyke and Bosworth.

Even if the insolubility of uncombined caseinogen is admitted to be true, as van Slyke and Bosworth claim, yet there remains the possibility that the so-called monobasic caseinogenate may be soluble in alkali chloride solution and insoluble in water, just as in the case of the alkaline earth caseinogenates.

No one has succeeded, so far as I know, in dissolving pure caseinogen (uncontaminated by base) in alkali solution in a proportion of $11{\cdot}5 \cdot 10^{-5}$ g. equivalent directly. Even when we triturate caseinogen in a mortar with

alkali solution for two or three hours, it is not easy to dissolve caseinogen in a proportion of $22{\cdot}5 \cdot 10^{-5}$. It seems hardly credible therefore that the solubility of caseinogen should be more than twice this value. It seems reasonable to ascribe the results obtained by van Slyke and Bosworth as well as by Robertson to the greater solubility of caseinogen in alkali chloride solution or to the solubility of so-called monobasic alkali caseinogenate in alkali chloride solution, if such a combination indeed exists. And the water-soluble combination of alkali and caseinogen should be accepted as containing $22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

Our experimental results described in the following pages appear to support this view.

Experiment 2. In this experiment, the solubility of caseinogen in pure alkali (NaOH) solution was compared with that of caseinogen in 0·5 % saline solution and that of caseinogen in alkali solution containing 0·5 % alkali chloride (NaCl).

The object of this experiment was to investigate whether the solubility of caseinogen in alkali is increased by the addition of alkali chloride to the alkali solution.

0·4 g. or 0·8 g. of caseinogen was accurately weighed and triturated in a glass mortar respectively with 5 cc. distilled water, 5 cc. 0·5 % NaCl solution, 5 cc. $N/500$ NaOH solution, 5 cc. $N/500$ NaOH solution in 5 % NaCl solution, very thoroughly for about 1·5 hours in each case, and then the mixtures were poured into centrifuge tubes. The mortar was washed with 45 cc. of distilled water in each case and the wash water was also added to the solutions in the tubes. They were then stoppered with cotton wool and were allowed to stand at room temperature (13°–15°) for 24 hours, being shaken from time to time, the air over the solution being changed often so that the CO_2 driven from the solution by the caseinogen was removed.

The solutions were then centrifuged, the supernatant fluid cautiously siphoned off, and the sediment washed carefully with absolute alcohol and ether dried over sodium and then dried and weighed.

By subtracting the obtained weight of sediment from the amount taken (0·4 or 0·8 g.) the amounts of caseinogen dissolved in these four solvents were determined. The results are given in Table IV. The loss of weight of caseinogen triturated with distilled water is apparently manipulative and must be taken into consideration also in the other cases.

Table IV.

Amount of triturated caseinogen. g.	Solvent 50 cc.	Weight of sediment. g.	Amount of caseinogen dissolved. g.
0·4	distilled water	0·3941	0·0059
0·4	0·5 % NaCl	0·3928	0·0072
0·4	$N/500$ NaOH	0·0246	0·3754
0·4	$N/500$ NaOH in 0·5 % NaCl	0·0000	0·4000
0·8	distilled water	0·7922	0·0078
0·8	0·5 % NaCl	0·7885	0·0115
0·8	$N/500$ NaOH	0·4070	0·3930
0·8	$N/500$ NaOH in 0·5 % NaCl	0·0236	0·7764

As will be seen from the table, 50 cc. of NaOH solution, when alkali chloride is added to it, dissolved 0·7764 g. of caseinogen, while the alkali solution alone dissolved only 0·3754–0·3930 g.; that is, the solubility is almost doubled in the former case. That this is not due to the ability of pure NaCl solution to dissolve caseinogen is evident since the amount of caseinogen dissolved in 0·5 % NaCl solution was only 0·0072–0·0115 g.

This experimental result suggests very strongly that the solubility observed by Robertson and Bosworth and by van Slyke is not the solubility of caseinogen in alkali solution but the solubility of caseinogen in alkali solution containing alkali chloride.

Thus it appears very probable that the alkali caseinogenate which contains $11{\cdot}4 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen or the monobasic alkali caseinogenate, as it is called by van Slyke and Bosworth, even if such a substance is admitted to exist, is not soluble in water and soluble only in alkali chloride solution.

As it is impossible to dissolve caseinogen in pure alkali solution in the direct way in larger proportion than $22{\cdot}5–25{\cdot}0^{-5}$ g. equivalent alkali per g. caseinogen it seems reasonable to assume that the alkali caseinogenate which is soluble in water must contain this percentage of alkali.

Calculated from this solubility value ($22{\cdot}5–25{\cdot}0 \cdot 10^{-5}$), the molecular weight of caseinogen is 4000–4400, one molecule of caseinogen containing one atom of phosphorus and one atom of sulphur.

More evidence must be produced before 8000–8800 can be admitted to be the true molecular weight of caseinogen.

C. The molecular and ionic concentration and the degree of dissociation of alkali caseinogenate of acid reaction.

We have seen in Exp. 1 that the osmotic concentration of alkali caseinogenate solution of neutral and alkaline reactions, is dependent upon the concentration of the alkali in which the caseinogen is dissolved, and that the amount of dissolved caseinogen does not affect in any appreciable degree the osmotic concentration between proportions of alkali to caseinogen from $180 \cdot 10^{-5}$ to $50 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

In Exp. 2, the molecular weight of caseinogen was shown to be 4000–4400, if calculated from the solubility of caseinogen in alkali solution. At any rate, the largest ionic weight of the protein radical in the alkali caseinogenate which is soluble in water must be 4000–4400, even if 8800 is ever proved to be the true molecular weight of uncombined caseinogen.

Now, if the alkali bound by caseinogen does not dissociate at all and no inorganic ions are present in the caseinogenate solution, and if polymerisation takes place in a caseinogenate solution of acid reaction while the caseinogen molecule is split successively into smaller protein ions by adding base, as Robertson [1918] has suggested, then the relationship between the osmotic

concentration of alkali caseinogenate solution and the concentration of the alkali in which the caseinogen was dissolved, must be maintained in caseinogenate solutions of acid reaction. Consequently we should expect to be able to obtain 4400 as the molecular weight of caseinogen by means of Barger's method when caseinogen is dissolved in alkali in the proportion of $22{\cdot}5 . 10^{-5}$ g. equivalent alkali per g. caseinogen. But it is quite clear that this expectation cannot be realised experimentally.

4400 will not be obtained by Barger's method for the molecular weight of the caseinogenate at the point of the maximum solubility of caseinogen in alkali solution, simply because the reaction of this caseinogenate solution is acid.

The fact that the reaction of alkali caseinogenate solution is acid when the percentage of base in caseinogenate is smaller than $50 . 10^{-5}$ g. equivalent indicates the presence of free H ions in the solution, that is the molecule of caseinogenate is dissociated into the protein radical and H ions.

The H ion concentration is enhanced from P_H 8·5 to P_H 7·0 when the proportion of alkali to caseinogen is decreased from $80 . 10^{-5}$ to $50 . 10^{-5}$ g. equivalent per g. It will be, therefore, very easily realised that many H ions are dissociated when an alkali solution is saturated with caseinogen. Moreover, the hypothesis that the alkali salt of such a strong acid as caseinogen does not dissociate in the solution at all, is very doubtful. Under these circumstances, we believe that the results obtained in the following experiment may not be very erroneous.

Experiment 3. In this experiment, the molecular and ionic concentration of alkali (NaOH) caseinogenate solution of acid reaction was determined by Barger's method.

Table V.

(*a*) NaOH = $N/50$, caseinogen = 5 %; alkali: caseinogen = $40 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/35–1/40 *M*.
The mean molecular ionic weight = 1875.

(*b*) NaOH = $N/50$, caseinogen = 6 %; alkali: caseinogen = $33{\cdot}3 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/30–1/35 *M*.
The mean molecular ionic weight = 1950.

(*c*) NaOH = $N/50$, caseinogen = 7 %; alkali: caseinogen = $28{\cdot}5 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/30 *M*.
The mean molecular ionic weight = 1750.

Table VI.

(*d*) NaOH = $N/33{\cdot}3$, caseinogen = 8·0 %; NaOH: caseinogen = $37{\cdot}5 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/20–1/25 *M*.
The mean molecular ionic weight = 1800.

(*e*) NaOH = $N/33{\cdot}3$, caseinogen = 10·0 %; NaOH: caseinogen = $30 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/17·5–1/20 *M*.
The mean molecular ionic weight = 1875.

(*f*) NaOH = $N/33{\cdot}3$, caseinogen = 10·8 %; NaOH: caseinogen = $27{\cdot}8 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/17·5–1/20 *M*.
The mean molecular ionic weight = 2025.

(*g*) NaOH = $N/33{\cdot}3$, caseinogen = 13·5 %; NaOH: caseinogen = $29{\cdot}6 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/15–1/17·5 *M*.
The mean molecular ionic weight = 2194.

As shown in Tables V and VI, the osmotic concentration of alkali caseinogenate solution of acid reaction is not dependent upon the concentration of alkali solution in which the caseinogen was dissolved, thus differing from caseinogenate solutions of neutral or alkaline reaction. The factor which conditioned the molecular and ionic concentration of the solution in this experiment was the amount of caseinogen dissolved. The osmotic concentration was almost parallel with the amount of caseinogen dissolved, and consequently the mean weight of the dissolved molecules and ions in the solution was calculated to be about 2000.

The objection might be raised that the result is due to impurities in the caseinogen. But such cannot be the case, because dialysis of the caseinogenate solution did not lower the osmotic concentration of the solution, as shown in the following experiment.

The impurities which may influence the osmotic concentration of the solution would be crystalloid substances having small molecular weights.

Colloids with large molecular weights would not change the osmotic concentration of the caseinogen solution, even though they might happen to be contained in the caseinogen employed. It would, therefore, be expected that dialysis of the caseinogen solution would remove most of any contaminating substance which could influence the osmotic concentration of the solution.

Experiment 4. Caseinogen solution was dialysed in a very thin collodion sack in a cold room for 48 hours, the distilled water outside the collodion tube being changed frequently.

The volume of the solution (0·3 g. caseinogen dissolved in $N/25$ NaOH solution) increased from 3·0 to 8·8 cc.

Table VII.

The molecular and ionic concentration after dialysis = 1/60–1/70 M.
The mean molecular ionic weight = 2206.
(The tubes in this experiment stood 32 hours after their preparation.)

Thus in every case, 2000 appears as the mean molecular and ionic weight of alkali caseinogenate when determined by Barger's method, if the proportion of alkali to caseinogen is smaller than $50 \cdot 10^{-5}$ g. equivalent per g. caseinogen.

As has been stated previously, the molecular weight of alkali caseinogenate when determined by calculation from the combining capacity, supposing the solubility of caseinogen in alkali to be $25\text{–}22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen, must be 4000–4400. We must therefore admit that one molecule of alkali caseinogenate is dissociated into two ions in solutions of acid reaction when the solutions are weak.

In the case of caseinogenate solutions of neutral or alkaline reaction, there may not exist any inorganic ions in the solution as Robertson suggests, the protein molecule being split into protein ions only, but in the case of alkali caseinogenate solutions of acid reaction, it is beyond doubt that the ions in

the solution are mostly those of the protein radical and hydrogen, since the reaction of the solution is acid.

Whether there are any alkali ions dissociated from the caseinogenate molecule, and whether there are any protein ions derived from the splitting of .CO.NH groups, is a very difficult problem to solve.

After I had finished these experiments, my attention was called to the paper of Plimmer and Bayliss [1906] in which they report that caustic soda of strong concentration (1 %) has the power of splitting off P_2O_5 from dissolved caseinogen. Though they admit from their experimental basis that "dilute alkali sufficient only to dissolve the caseinogen causes no separation of soluble P_2O_5," and, as a matter of fact, they themselves employed caustic soda as the solvent of caseinogen in their experiments, and though furthermore the fact that I have obtained quite the same results in my Exp. 1 as those obtained by Robertson and Burnett employing alkaline earths and other alkalies than NaOH, affords clear evidence that the specific decomposing action of caustic soda of strong concentration found by Plimmer and Bayliss was not taking place in my experiments, still I thought it might be of some use to test experimentally whether similar results can be secured when other alkalies than NaOH are used as solvents. Thus I performed a few experiments employing NH_4OH as solvent. The results are as follows.

(I) $NH_4OH = M/50$. Caseinogen = 4 %. Alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = $1/47{\cdot}5$ *M*.

(II) $NH_4OH = M/50$. Caseinogen = 6·0 %. Alkali: caseinogen = $33{\cdot}3 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = $1/33{\cdot}3$ *M*.

(III) $NH_4OH = M/33{\cdot}3$. Caseinogen = 10·8 %. Alkali: caseinogen = $27{\cdot}8 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = 1/15 *M*.

These results are quite the same as observed when NaOH was used as solvent of caseinogen.

Summary.

In the present paper, the solubility of caseinogen and the osmotic concentration of alkali caseinogenate solutions have been investigated, employing Barger's method of determining molecular weights, and it has been found that:

1. The solubility of caseinogen in alkali solution containing a certain amount of alkali chloride, is almost twice that of caseinogen in pure alkali solution.

2. The molecular and ionic concentration of alkali caseinogenate solutions of neutral and alkaline reaction is conditioned by the concentration of the alkali solution in which the caseinogen is dissolved.

3. The osmotic concentration of alkali caseinogenate solutions of acid reaction is dependent upon the amount of dissolved caseinogen, and the mean weight of the ions in the solution is about 2000.

As a result of these experiments, it is suggested that the solubility of caseinogen in alkali solution obtained by Robertson, as well as by van Slyke

and Bosworth, seems very probably to have been that of caseinogen in alkali solution containing alkali chloride. The true solubility of caseinogen in pure alkali, therefore, must be $22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

The molecular weight of alkali caseinogenate which is soluble in distilled water must be 4000–4400, and one molecule of caseinogenate seems to be dissociated in such dilute solutions as were investigated (1/15–1/70 *M*) mainly into two ions, consisting principally of H-ions and protein ions, beside the ions of the alkali and split protein.

In conclusion, I wish to express my thanks to Prof. C. J. Martin who supplied me with material and apparatus.

REFERENCES.

Plimmer and Bayliss (1906). *J. Physiol.*, **33**, 453.
Robertson (1909). *Univ. of California, Publ. Physiol.*, **3**.
—— (1918). *The Physical Chemistry of Proteins.*
Robertson and Burnett (1909). *J. Biol. Chem.*, **6**, 105.
van Slyke and Bosworth (1913). *J. Biol. Chem.*, **14**, 203, 207, 211.
Yamakami (1920). *Biochem. J.*, **14**, 103.